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Probing the expression and function of the P2X7 purinoceptor with antibodies raised by genetic immunization

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Abstract

The cytolytic P2X7 purinoceptor is widely expressed on leucocytes and has sparked interest because of its peculiar ability to induce a large nonselective membrane pore following treatment of cells with ecto-ATP. Antibodies raised against synthetic P2X7 peptides generally work well in Western-Blot analyses but fail to recognize the native protein on the cell surface. Genetic immunization is a useful technique to raise antibodies directed against proteins in native conformation. Using this technique we have generated highly specific polyclonal (rabbit) and monoclonal (rat) anti-P2X7 antibodies that readily detect mouse P2X7 on the surface of living cells by immunofluorescence analyses and flow cytometry. Binding of these antibodies to P2X7 is reduced within seconds after treatment of cells with ATP, suggesting that ligand binding induces a conformational shift and/or the shedding of P2X7. By site directed mutagenesis we have mutated three conserved arginine residues (R294A, R307A, R316A) in the extracellular loop of P2X7 near the second transmembrane region. Each of these mutations results in loss of ATP response. FACS and immunoblot analyses reveal that the R294A mutant is expressed at higher levels than wild-type P2X7 in transfected cells, whereas the R307A and R316A mutants are barely detectable because there is no or very little protein synthesis of these constructs. In accord with its resistance to ATP-induced activation the R294A mutant is not down-modulated from the cell surface by ATP-treatment.

Keywords: Monoclonal antibodies; Purinoceptor; Genetic immunization

1. Introduction

Following its release from cells, ATP functions as an extracellular signaling molecule [1]. Molecular cloning has identified seven ATP-gated ion channels (P2X purinoceptors) and eleven ATP-binding G-protein coupled receptors (P2Y purinoceptors) [2,3]. Among these, P2X7 is widely expressed on leucocytes and has sparked interest because of its peculiar ability to induce a large nonselective membrane pore, causing membrane blebbing and apoptosis (Fig. 1) [2]. Activation of P2X7 either by high concentrations of ecto-ATP or by NAD-dependent ADP-ribosylation induces calcium flux, exposure of phosphatidyl serine, DNA fragmentation and uptake of propidium iodide [4,5]. A number of naturally occurring functionally impaired P2X7 mutants have been described in both the human and the mouse [6–9].

Site directed mutagenesis has been used to pinpoint amino acid residues involved in ATP-binding and other functionally relevant residues of P2X7 [10–13]. These studies are fraught with the problem of distinguishing direct effects of mutations on ligand-binding from mutations that affect the level of cell surface expression of P2X7. A number of commercially available

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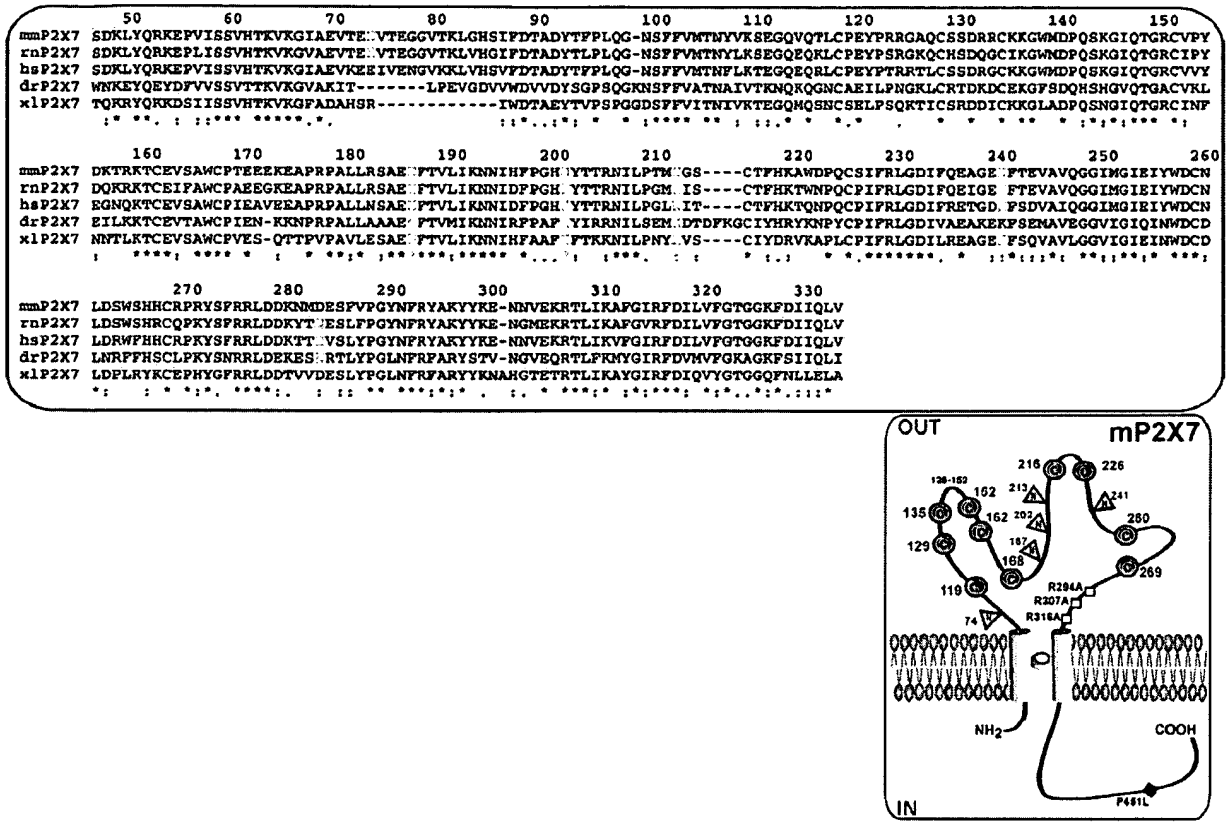

Fig. 1. Schematic diagram of mouse P2X7 and the mutants analyzed in this study. The conserved arginine residues that were mutated to alanine in this study are highlighted in yellow in the schematic diagram of mouse P2X7 and in the amino acid sequence alignment of the extracelluar loops of P2X7 from human (hs), mouse (mm), rat (rn), frog (xl), and zebrafish (dr). Identical and conserved amino acids are marked below the alignment by asterisks and colons, respectively. Conserved cysteine residues are in red, potential N-linked glycosylation sites are in green, and the residues (136–152) of mouse P2X7 used for generating the "anti-P2X7 extracellular" antibody (Alomone labs, Jerusalem) are in blue. The P451L allelic variant in the C-terminal cytosolic tail of P2X7 is marked by a blue diamond in the schematic diagram.

anti-purinoceptor antibodies were raised by immunization with synthetic peptides derived from the known amino acid sequence. These antibodies generally work well in Western blot analyses but often fail to recognize the native protein on the cell surface. Such antibodies can be used successfully for monitoring overall purinoceptor expression in a population of cells but cannot be used to assess cell surface expression on individual cells. Genetic immunization is a very useful technique to raise antibodies directed against proteins in native conformation [14,15]. Here we have employed this technique to generate highly specific polyclonal and monoclonal anti-P2X7 antibodies and illustrate the utility of these antibodies for probing the expression and function of P2X7.

2. Materials and methods

2.1. Cloning of expression vectors and cell transfections

The full length cDNA sequence for mouse P2X7 was PCR amplified from mouse splenocyte cDNA and cloned under control of the CMV promoter in the pCDNA6.1 vector (Invitrogen). The R294A, R307A, and R316A mutants were generated by site directed PCR-mutagenesis using the quickchange system (Stratagene). An expression construct for nuclear green fluorescent protein was generated by cloning the DNA binding domain of the LKLF transcription factor [16] as a C-terminal fusion protein into the pEGFP-Nl vector (Clontech). Expression constructs were transfected into CHO and HEK cells (5 μg P2X7 and/or 0.5 μg eGFP_LKLF-DBD per T25 flask containing 2×10^6 cells) with the JetPei transfection reagent (Polyplus transfection). For stable transfection of HEK cells, plasmids were linearized with *Ssp*I before transfection and stable transfectants were selected by cultivating cells in the presence of blasticidin.

2.2. Genetic immunization and production of monoclonal antibodies

For gene gun immunizations, the P2X7-expression construct was conjugated to 1 μm gold particles (Bio-Rad) and rabbits and rats were immunized by ballistic DNA immunization with a pressure setting at 400 psi.

Animals received four immunizations in three-six week intervals with 12 (rabbit K1G) and eight (rats) shots of plasmid-conjugated gold particles per immunization (1 μg DNA/mg gold/shot). Serum samples were obtained 10 d post-immunization. For monoclonal antibody production, animals received a final boost with P2X7-transfected HEK cells (2×10^6 cells in 200 μl PBS i.v.) 3 d prior to sacrifice. Splenocytes were fused to Sp2/0 myeloma cells and screened for production of P2X7-specific antibodies by immunofluorescence analysis using untransfected and P2X7-transfected CHO cells as targets. Specificity and reactivity of antibodies was confirmed by flow cytometry using FITC- or PE-conjugated anti-rabbit immunoglobulin and anti-rat immunoglobulin antibodies (Dianova) (see below). Two hybridomas (Hano44 and Hano43) that showed prominent specific staining of P2X7-transfected cells were selected for further analyses and were subcloned by limiting dilution. Hano44 and Hano43 were isotyped as rat IgG2b/κ using a rat immunoglobulin isotyping kit according to the manufacturer's (BD Bioscience) instructions. Antibodies were purified by affinity chromatography on Protein G sepharose (Pharmacia), and conjugated to Alexa488 according to the manufacturer's (Molecular Probes) instructions.

2.3. Immunofluorescence and FACS analyses

CHO cells were cotransfected with expression constructs for wild-type mouse P2X7 and a nuclear GFP (5 and 0.5 μg/2×10^6 cells, respectively). Cells were passaged onto 96 well plates (4×10^5 cells/well) 6 h post-transfection. 20 h after transfection cells were fixed for 10 min in 2% paraformaldehyde and then stained with rabbit K1G immune serum (1:500) induced by P2X7 cDNA-immunization or with rabbit anti-P2X7 peptide antibody (1:500) raised against the conserved peptide KKGWMDPQSKGIYTGRC in the extracellular loop of P2X7 (residues 136-152) (Alomone labs). Bound antibody was detected with PE-conjugated anti-rabbit IgG (1:100) (Dianova). Cell nuclei were counterstained with Hoechst 33342. Cells were analyzed with a Zeiss Axiovert 25 microscope equipped with the Canon PowerShot G2 digital camera and appropriate filters for visualizing Hoechst stain (Zeiss filter set 01), green fluorescent protein (Zeiss filter set 10) and PE fluorochrome (Zeiss filter set 15). Photographs were taken at 40× magnification with fixed camera settings (digital zoom:4.3×, aperture:5.0, exposure time: 2 s for GFP and PE, 1/8 s for Hoechst 33342). GFP and PE images were merged using Adobe Photoshop software. For FACS analyses, cells (5×10^5/100 μl) were stained either directly with Alexa-488 conjugated antibodies (1 μg) or with K1G immune serum (1:2000) followed by PE-conjugated anti-rabbit IgG (1:100, Dianova). Gating was perfomed on living cells on the basis of propidium iodide exclusion.

2.4. Immunoblot analyses

Cells (10^7/ml) were lysed in PBS, 1% Triton-X100, 1 mM AEBSF (Sigma) for 20 min at 4 °C. Insoluble material was pelleted by centrifugation and soluble proteins (5×10^5 cell equivalents/lane) were size fractionated on precast SDS-PAGE gels (Invitrogen) and blotted onto PVDF membranes. P2X7 was detected with rabbit anti-peptide 136–152 antibody (1:1000) (Alomone) and PO-conjugated anti-rabbit IgG (1:5000) using the ECL-system (Amersham).

3. Results

Antibodies raised by genetic immunization of rabbits and rats with P2X7 cDNA expression constructs were assayed for reactivity and specficity by indirect immunofluorescence staining of CHO cells 24 h after cotransfection with cDNA expression constructs for P2X7 and nuclear green fluorescent protein. By this time, transfected cells were clearly distinguishable from untransfected cells by virtue of their green fluorescent nuclei (Fig. 2). Transfected cells also showed bright cell surface staining following incubation with polyclonal K1G rabbit (Fig. 2) or mAbs Hano44 and Hano43 (not shown) and appropriate PE-conjugated secondary antibodies. These antibodies did not show any detectable reactivity with untransfected cells or with cells transfected with human P2X7 (not shown). A commercially available anti-P2X7 peptide antibody directed against a peptide in the extracellular loop of P2X7 that recognizes denatured P2X7 in immunoblot analyses (see below) showed only little if any staining of P2X7-transfected cells (Fig. 2).

We mutated three conserved arginine residues (R294, R307, and R316, see Fig. 1) in the extracellular loop of P2X7 just upstream of the second transmembrane region. HEK cells transfected with wild-type P2X7 showed typical P2X7-mediated responses to treatment with ecto-ATP, including exposure of phosphatidylserine, membrane blebbing and uptake of the DNA-staining dye YOPRO-1 [6]. In contrast, untransfected cells or cells transfected with the generated R294A, R307A, or R316A mutants did not show any detectable responses (not shown). Cells were assayed for cell surface expression of P2X7 by flow cytometry with anti-P2X7 polyclonal K1G antibodies and PE-conjugated anti-rabbit immunoglobulin 40 h after transfection and 40 d after selection with blasticidin (Fig. 3). The results revealed moderate staining of wild-type transfectants, and cells transfected with the functionally impaired natural P451L variant showed similar cell surface staining levels. Cells transfected with the R294A mutants showed higher cell surface expression, whereas cells transfected with the R307A and R316A mutants showed only very faint if any staining. Similar results were obtained with mAb Hano43 (Fig. 3).

Immunoblot analyses were performed to assess overall expression levels of wild-type and mutant P2X7 (Fig. 4). The results are in accord with those obtained by flow cytometry, i.e., a stronger band is seen in lysates of R294A transfectants (lane 1) than in wild-type transfectants or P451L transfectants (lanes 4 and 5). In contrast very faint if any specific bands are seen in lysates of R307A and R316A mutants (lanes 2 and 3). These findings indicate that the impaired responses of the R307A and R316A transfectants to ATP most likely are a consequence of their reduced levels of cell surface expression, which in turn most likely are due to reduced rates

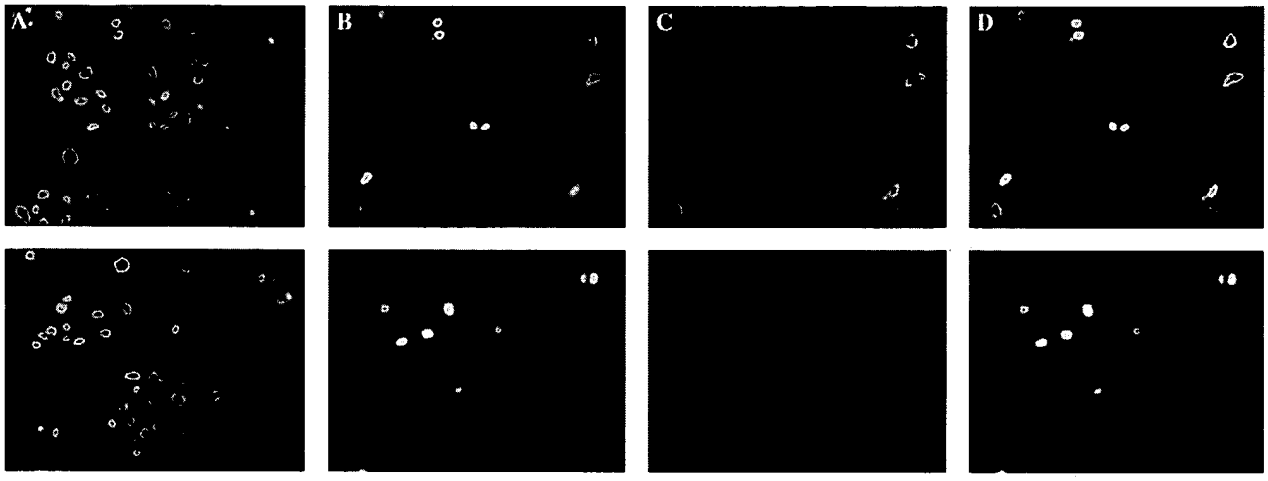

Fig. 2. Immunofluorescence analyses of CHO cells following transient cotransfection with expression constructs for mouse P2X7 and nuclear green fluorescent protein. CHO cells were cotransfected with expression constructs for wild-type mouse P2X7 and nuclear GFP. Twenty hours after transfection cells were stained with rabbit anti-P2X7 K1G antiserum (top panels) or rabbit anti-P2X7 peptide 136–152 anti-serum (bottom panels) followed by PE-conjugated anti-rabbit IgG. Cell nuclei were counterstained with Hoechst 33342. Cells were analyzed with a Zeiss Axiovert 25 microscope equipped with filters for visualizing Hoechst dye (A), green fluorescent protein (B) and PE fluorochrome (C). (D) Merged images of GFP and PE stainings (b + c).

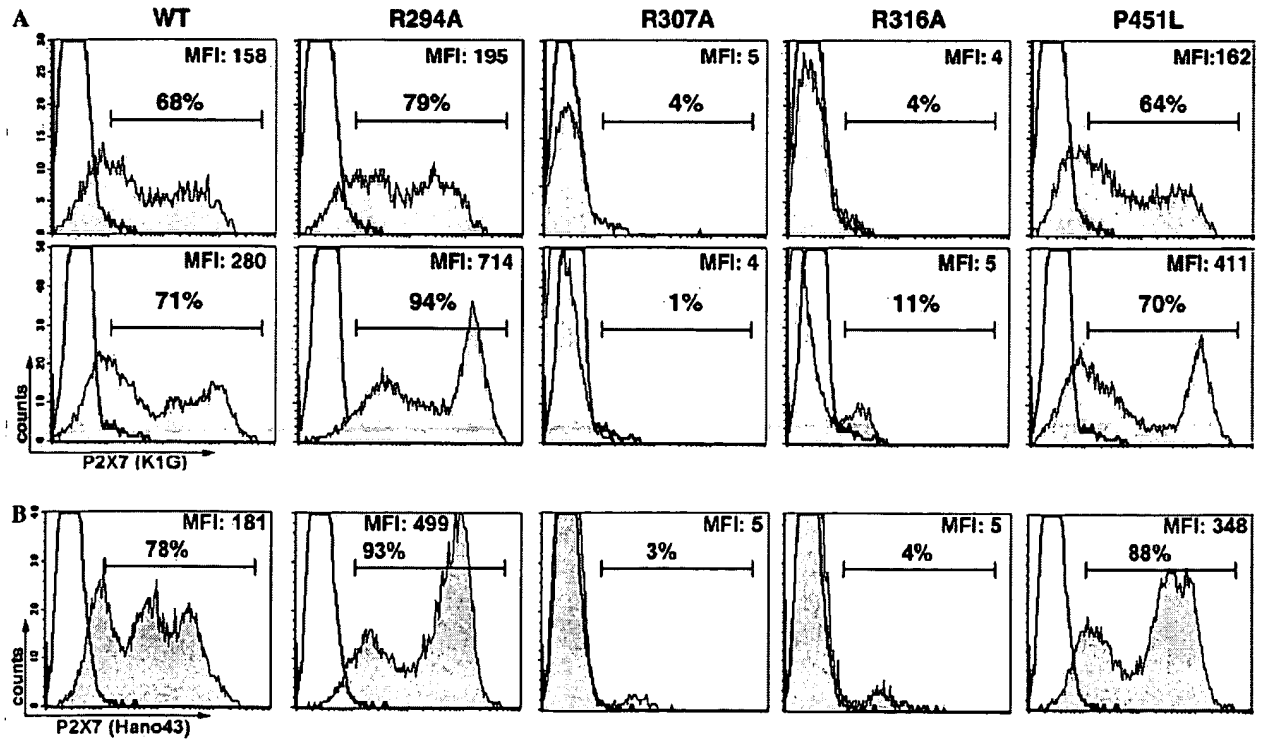

Fig. 3. FACS analyses of P2X7-expression by transfected HEK cells HEK cells were transfected with expression constructs for wild-type P2X7, naturally occurring allelic variant P451L, and site directed mutants R294A, R307A, and R316A. At 40 h (top) and 40 d (bottom) post transfection cells were stained with anti-P2X7 K1G anti-serum and PE-conjugated anti-rabbit IgG (A) or with anti-P2X7 mAb Hano43 and PE-conjugated anti-rat IgG (B) before FACS analyses (shaded histograms). Control stainings were performed with untransfected cells (open histograms).

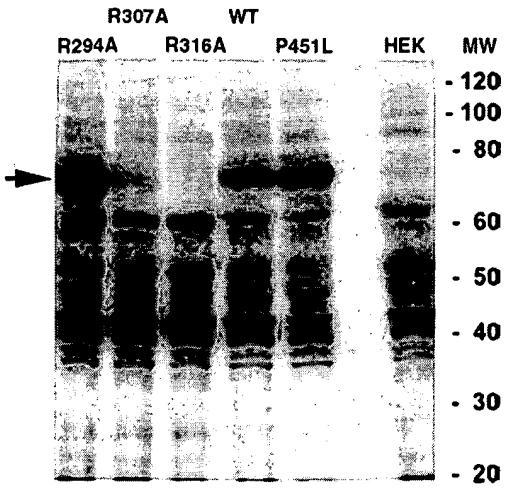

Fig. 4. Immunoblot analyses of P2X7-expression by transfected HEK cells Untransfected and stably transfected HEK cells were solubilized 40 d post transfection with 1% Triton-X-100. Proteins in cell lysates were size fractionated by SDS–PAGE and subjected to immunoblot analyses using rabbit anti-P2X7 peptide 136–152 antibodies. Bound antibodies were detected with peroxidase conjugated secondary antibody and the ECL system.

of synthesis and/or enhanced rates of degradation. In contrast, the impaired response of the R294A transfectants to ATP is not due to lack of cell surface expression or impaired folding of the mutant purinoceptor, and suggests that this mutant may be incapable of binding ATP.

Intriguingly, treatment of wild-type P2X7 transfectants with high doses of ATP consistently resulted in reduced staining of cells with both, polyclonal K1G and monoclonal Hano44 and Hano43 antibodies (Fig. 5). Comparative dose response and kinetic analyses of this phenomenon revealed rapid and dose

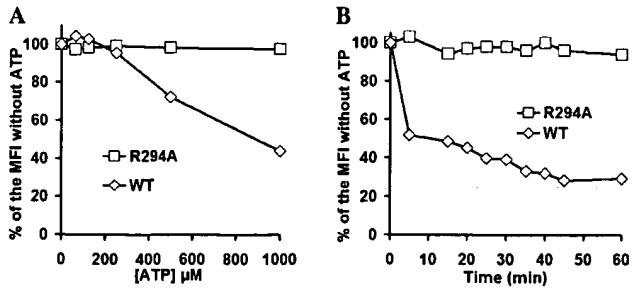

Fig. 5. Dose response (A) and kinetics (B) of modulation of P2X7 cell surface expression by transfected HEK cells following treatment with ATP. (A) HEK cells stably transfected with wild-type P2X7 (diamonds) or with the R294A mutant (squares) were treated with the indicated concentrations of ATP for 30 min, washed, and then subjected to FACS analyses with Alexa^{488}-conjugated mAb Hano43. Gating was performed on living cells on the basis of propidium iodide exclusion. (B) Stably transfected HEK cells were treated with lmM ATP for the times indicted, washed, and then subjected to FACS analyses with Alexa^{488} conjugated mAb Hano43. The results are expressed as the percentage of the mean fluorescence intensity (MFI) of cells in the absence of ATP. Gating was performed on living cells.

dependent modulation of P2X7 in case of cells transfected with wild-type P2X7 but not in cells transfected with the R294A mutant (Fig. 5). The findings further illustrate the loss of functionality of the R294A mutant and underscore the utility of the P2X7-antibodies for monitoring the functionality of P2X7 mutants.

4. Discussion

Using genetic immunization, we have raised highly specific anti-P2X7 polyclonal and monoclonal antibodies. With these antibodies we show that the naturally occurring P451L allelic variant of the P2X7 receptor as carried by C57BL/6, DBA and other strains of mice [6] is efficiently expressed at the cell surface. The R294A mutant that was generated by site directed mutagenesis showed elevated cell surface expression levels but did not respond to ATP, whereas the R307A and R316A mutants showed little if any cell surface expression, reflecting reduced overall protein levels of these mutants. Treatment of P2X7-transfectants with ATP resulted in rapid down-modulation of detectable cell surface P2X7 in case of wild-type P2X7 but not in case of the R294A mutant, indicating that binding of ATP induces a change in conformation of P2X7 that affects the epitope(s) of the antibody binding site(s) and/or induces shedding/endocytosis of the receptor. The fact that down-modulation is not reversed after washing away the ATP is more compatible with shedding/endocytosis, in line with a recent report describing the ATP-stimulated internalization of human P2X7 in transfected HEK cells [17]. Our findings underscore the utility of genetic immunization for generating highly specific polyclonal and monoclonal antibodies directed against proteins in native conformation [14,15]. These antibodies are valuable tools for assessing the expression levels of the native protein by immunofluorescence analyses and flow cytometry, i.e., assays in which anti-peptide antibodies often fail. The P2X7-specific antibodies described here provide useful tools for further characterization of the structure and function of this intriguing nucleotide-gated ion channel.

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performed the immunofluorescence analyses shown in Fig. 2. FR performed the site-directed mutagenesis of P2X7. SA made the P2X7 transfectants and performed the experiments shown in Figs. 3–5.

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